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14. ABSTRACT

Loss of epithelial architecture is thought to be an early event in carcinoma. The mechanism by which oncogenes disrupt epithelial architecture is poorly understood. Previous results from our lab have shown that ErbB2, an oncogene correlated to poor clinical prognosis in breast cancer, can disrupt epithelial cell polarity. My research is aimed at investigating how activation of ErbB2 disrupts epithelial cell polarity. Activation of ErbB2 is known to induce phosphorylation of five tyrosines in its cytoplasmic tail. Using ErbB2 autophosphorylation site mutants I investigated whether a particular tyrosine residue mediates the ErbB2-induced changes in epithelial cell polarity. In mammary epithelial cells grown as three-dimensional (3D) structures, Y1144, Y1201, Y1226/7 and Y1253 were all sufficient to disrupt the structural architecture when activated during the process of morphogenesis. However, if these same sites were activated after structures were fully formed they were unable to disrupt the acinar architecture. It therefore appears that in fully organized three-dimensional structures more than one tyrosine site is needed to disrupt architecture, whereas in developing structures one site is sufficient.

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Introduction

The aim of this grant is to investigate the mechanism by which ErbB2, an oncogene commonly overexpressed in breast cancer, regulates the Par complex, one of the three known complexes important in establishing cell polarity [1]. In order to address this question we chose to investigate the contribution of specific autophosphorylation sites to ErbB2-induced changes in epithelial cell polarity.

Our lab uses a unique ErbB chimeric receptor, in which both the extracellular and transmembrane domains have been replaced with corresponding domains from the p75 low affinity NGFR receptor. Dimerization is mediated by a small molecule ligand that binds to the FKBP domain which is fused to C-terminal region of chimeric ErbB2 [2]. In contrast to constitutively active versions of ErbB2, the inducible system offers us the advantage to activate ErbB2 in polarized proliferation-arrested epithelial cells and to investigate the effect of ErbB2 activation on cell polarity [3].

Previous studies have identified five tyrosines in ErbB2's carboxy terminal tail that are autophosphorylated upon ErbB2 dimerization. It was shown that ErbB2 receptor molecules with at least one active autophosphorylated tyrosine, Y1144, Y1201, Y1226/1227 or Y1253, can promote transformation of fibroblasts [4]. I have generated two classes of autophosphorylation site mutants, a) the "add-backs" - a series where all but one tyrosine are active and b) the deltas - a series where only one tyrosine is active.

In order to further investigate the effect of these mutants I am using MCF-10A, a human breast epithelial cell line known to form polarized three-dimensional (3D) acini-like structures in culture [6]. My current studies suggest that Y1144, Y1201, Y1226/1227 and Y1253 are all capable of disrupting architecture in developing structures but, unlike wild type ErbB2, are not capable of disrupting the architecture of fully formed structures.

Body

Using the chimeric receptor as "wild type" ErbB2 I have created two distinct series of mutations in order to investigate the role of individual autophosphorylated tyrosines and ErbB2 localization in its ability to disrupt cell polarity.

I previously reported that Y1144 was required for ErbB2 induced disruption of epithelial cell polarity in Madin Darby Canine Kidney (MDCK) cells, as was suggested by clones that I had studied. However, further analysis of the clones found that the low levels of expression of the chimera in these cells gave a very weak phenotype, even with the activation of ErbB2. Therefore it was unclear if the effect seen in the clones was due to slight variation in expression level, not detectable by western blotting. To avert this problem we FACS sorted pools of MDCK cells expressing the different clones. However, technical difficulties led to variable expression levels within the FACS sorted populations. Because of the differences in expression level, no conclusions could be made from these cells.

In order to continue the structure function study we chose to express the autophosphorylation site mutations in an alternate cell line. The requirements for this cell line were that it be a non-transformed epithelial cell line which can polarize in cell culture. Of the many mammary cell lines available to us, very few are non-transformed. However, MCF10As are a mammary epithelial cell line derived from fibrocystic tissue. This non-transformed cell line is capable of forming polarized 3D acini-like structures in culture. As previously shown by our lab, activation of ErbB2 induces proliferation, protects against apoptosis and disrupts the organization of these structures. As such this cell line is ideally suited to our needs. We therefore expressed the different mutations in MCF10As. FACS sorting of these cells resulted in pools of cells with very similar expression, which allows for easy comparison of the mutant phenotypes.

Investigate the contribution of specific autophosphorylation sites

To investigate the roles that the five autophosphorylated tyrosines play in the disruption of epithelial cell polarity, I constructed two series of mutations. The "add-back" mutations have four of the five sites mutated to phenylalanine, thereby inactivating those sites and leaving only one site active. The add-backs can be used to determine which site(s) are sufficient for disrupting epithelial cell polarity. The complimentary set of mutants, the delta mutations, has only one site inactivated by a phenylalanine mutation, thus allowing for the determination of which, if any, sites are necessary.

I initially screened the delta mutations for ability to disrupt polarity in MDCK cells, but found that there was no significant difference between the delta mutations. This suggested that none of these tyrosines are necessary for disrupting the epithelial cell architecture. Therefore, I chose to study the add-back mutations in MCF-10As (Figure 2).

MCF10As are capable of forming 3D acini-like structures in culture, similar to those found in breasts. The fact that these acini develop in culture and then become growth arrested and polarized acini allows for the investigation of two processes. First, what is necessary to disrupt the architecture of a developing acinus? A developing acinus undergoes a program which maintains the acinus in a spherical shape and clears the internal cells to form a hollow sphere. In order to disrupt the architecture of a developing acinus, this process must be inhibited. In a fully developed acinus, where this program has already been successfully executed there is the need to reinitiate a remodeling process in cells which are now programmed to maintain their organization relative to their neighbors. Therefore activation of ErbB2 during acinar development addresses whether ErbB2 can disrupt the standard morphogenetic program of an acini, while activation of ErbB2 in a fully formed acinus addresses the ability of ErbB2 to reinitiate a remodeling program in already polarized cells.

Previous studies in our lab have shown that ErbB2 is capable of disrupting the architecture of both developing and fully formed acini. I have used both of these assays to investigate the differences between the autophosphorylated tyrosine site mutations.

Single tyrosines are sufficient to disrupt the architecture of developing three-dimensional structures.

To determine if any individual tyrosine site can disrupt the morphogenetic program, the mutant ErbB2 constructs expressed in MCF10As were stimulated on day 4 after plating and monitored until day 12. A construct lacking all 5 tyrosine sites, YPD, did not dramatically disrupt acinar architecture in response to the stimulus, however, Y1144, Y1201, Y1226/1227 and Y1253 were all sufficient to induce multiacinar structures (Figure 3). This suggests that each individual tyrosine is capable of disrupting an ongoing morphogenesis program.

Single tyrosine sites are not sufficient to disrupt the architecture of fully formed three-dimensional structures.

To determine if individual tyrosines were also capable of disrupting architecture in fully formed acini, the mutants expressed in MCF10A cells were stimulated in 3D cell culture after acini had fully formed. Four days after stimulation wild type ErbB2 had induced multiacinar structures, disrupting the normal architecture. Although quantitation still remains to be done, it appears that no single tyrosine site was able to disrupt the cellular architecture. This suggests that additional constraints placed on cell organization and growth in fully formed acini are overcome only by cooperation between two or more of ErbB2's tyrosine sites. In order to further explore which sites might be cooperating to induce the changes in architecture we will use the delta mutations, as well as mutations that we have created containing 2-3 active tyrosine sites.

1c. ErbB2 interaction with Par6.

Other members of our lab have recently shown that ErbB2 coimmunoprecipitates with Par6, a key regulator of cell polarity. This interaction appears to be key to ErbB2's ability to regulate cell polarity (unpublished data). In order to further investigate the role of the tyrosine sites in the regulation of cell polarity, I have attempted to repeat the Par6-ErbB2 coimmunoprecipitation. However, none of the antibodies currently available to us are capable of detecting endogenous levels of Par6 in a western blot. Although I can co-immunoprecipitate ErbB2 with Par6 the lack of a Par6 western blot to control for the Par6 immunoprecipitation makes the results of these immunoprecipitations difficult to interpret. For this reason, I am in the process of overexpressing a flag-tagged Par6 in the various cell lines made during the course of this investigation. The advantage of using the flag-tagged Par6 is that we have antibodies in the lab that recognize the flag tag, which will make it possible to determine how much Par6 was pulled down each immnunoprecipitation. This control will allow us to better interpret the data we obtain and determine which of these sites is regulating cellular architecture through the Par6-ErbB2 interaction.

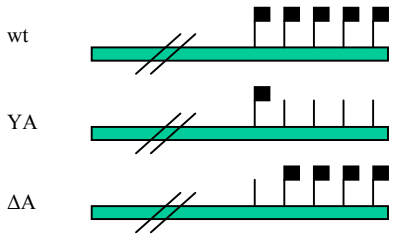

Figure 1: Examples of Y and Δ mutants. In Y mutants all but one of the autophosphorylation sites is mutated to phenylalanine, whereas in delta mutants 4 autophosphorylation sites remain intact and only one is mutated to phenylalanine.

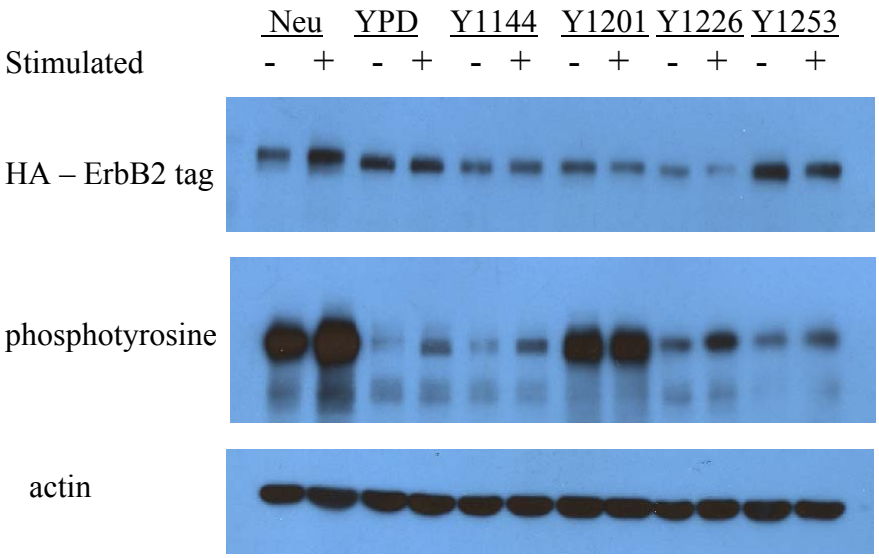

Figure 2. "Add-back" mutations expressed in MCF10As are inducibly phosphorylated. MCF10A cells expressing either wild type or one of the mutations were grown in plastic dishes for four days, or until confluent. The cells were starved overnight and then stimulated for 20 minutes, after which time they were lysed. 50ug of lysate were run out on an SDS-PAGE gel and then blotted for phosphotyrosine, to look at activation of the receptors. The blot was then stripped and reprobed for either HA, which tags the chimeric receptor, or actin,, which serves as a loading control.

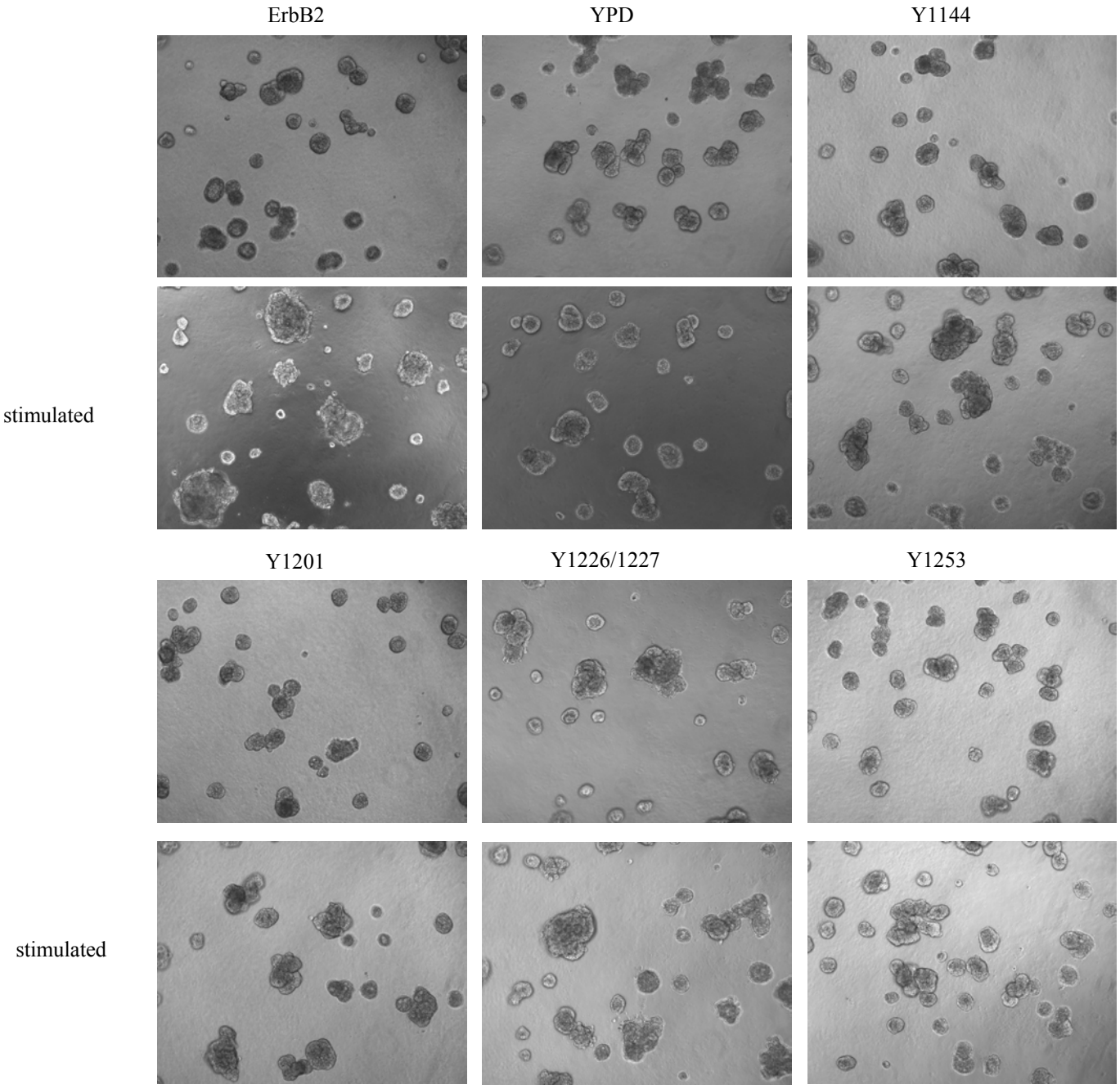

Figure 3. Individual tyrosine sites are capable of disrupting architecture in developing acini. MCF10A cells expressing chimera with either wild type ErbB2, a tyrosine phosphodeficient (YPD) mutants or individual add-back mutations were grown for 4 days in 3D culture. On day 4, when the acini were still undergoing proliferation and morphogenesis, the cells were stimulated with dimerizer to activate the overexpresed receptors. Images were taken on day 12, 8 days after stimulation, and analyzed for the presence of disorganized multiacinar structures.

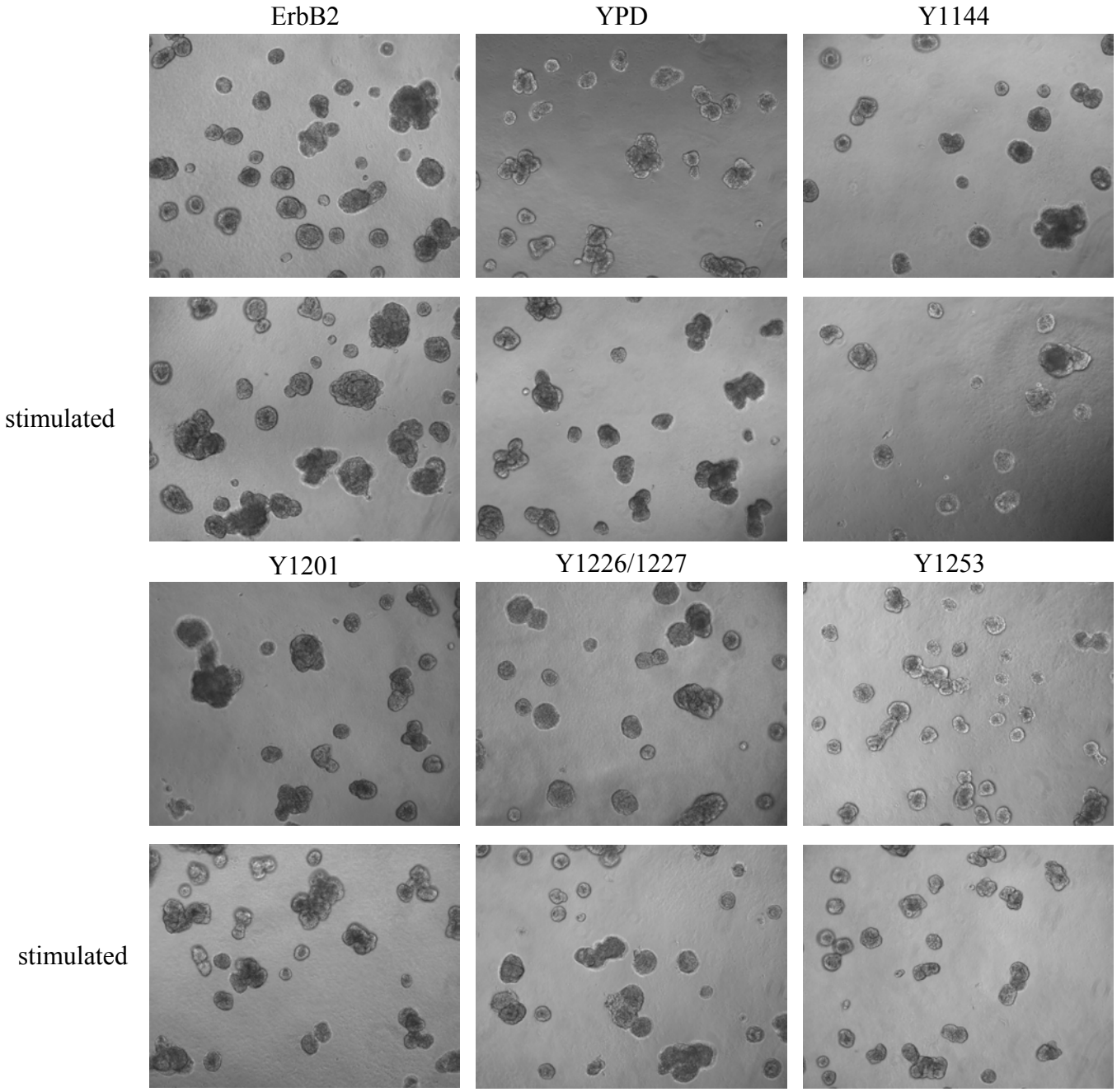

Figure 4. Wild type ErbB2, but not individual tyrosine sites, is capable of disrupting architecture in fully formed acini. MCF10A cells expressing chimera with either wild type ErbB2, YPD or individual add-back mutations were grown for 12 days in 3D culture. On day 12, after cells have stopped proliferating and are have well established organization, the cells were stimulated with dimerizer to activate the overexpresed receptors. Four days later images were taken and analyzed for the presence of disorganized multiacinar structures.

Key Accomplishments

- determination that none of the delta mutations dramatically affect ErbB2's ability to disrupt epithelial cell polarity.
- determination that tyrosines 1144, 1201, 1226/1227 and 1253 are all capable of disrupting the three-dimensional architecture in MCF10A cells when activated during the morphogenesis process of these structures.
- determination that tyrosines 1144, 1201, 1226/1227 and 1253 are not capable of changing the architecture of fully formed thee dimensional structures to the extent that wild type ErbB2 can disrupt the architecture.

Reportable Outcomes

- Lucs, Alexandra and Muthuswamy, Senthil (2005) Structure/Function Study of ErbB2 *Era of Hope – Department of Defense Breast Cancer Program Meeting.* Philadelphia, PA

- Lucs, Alexandra and Muthuswamy, Senthil (2005) Structure/Function Analysis of ErbB2's Ability to Disrupt Epithelial Apical-Basal Cell Polarity *American Society of Cell Biology Annual Meeting.* San Francisco, CA

Conclusion

The ErbB2 tyrosine sites 1144, 1201, 1226/7 and 1253 are all sufficient to disrupt cellular architecture in cells undergoing the process of morphogenesis. However none of these sites is sufficient to disrupt architecture of fully formed 3D acini. For this reason it appears that the additional constraints put on the growth and morphogenesis of a cell within a fully formed acini can only be overcome by a combination of these tyrosine sites. In the course of this investigation we have developed a number of mutants which have various combinations of tyrosine sites that we will use in order to further investigate which sites are necessary cause disruption of architecture in mature acini.

Although the individual sites have not clearly separated in their ability to regulate cellular architecture, we have used the mutations developed during this research to investigate the roles these tyrosines play in other aspects of the ErbB2 phenotype. Interestingly, preliminary results from these studies suggest that the tyrosines do have distinct phenotypes in the regulation of apoptosis as well as interactions with the growth factor TGFβ. Therefore as we continue to investigate how ErbB2 regulates cellular architecture, we will also use the tools developed during this grant to elucidate the regulation of other ErbB2 induced phenotypes.

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